

AWARD NUMBER: W81XWH-14-C-0084

TITLE: Deglycosylated filovirus glycoproteins as effective vaccine immunogens

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REPORT DATE: November 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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REPORT DOCUMENTATION PAGE		Form Approved OMB No. 0704-0188	
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1. REPORT DATE November 2015	2. REPORT TYPE Final	3. DATES COVERED 1 May 2014 - 31 Aug 2015	
4. TITLE AND SUBTITLE Deglycosylated filovirus glycoproteins as effective vaccine immunogens		5a. CONTRACT NUMBER W81XWH-14-C-0084	
		5b. GRANT NUMBER	
		5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Wendy Maury  E-Mail:wendy-maury@uiowa.edu		5d. PROJECT NUMBER	
		5e. TASK NUMBER	
		5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Iowa, Iowa City, IA 52242		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSOR/MONITOR'S ACRONYM(S)	
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT  Approved for Public Release; Distribution Unlimited			
13. SUPPLEMENTARY NOTES			
14. ABSTRACT <p>Viruses within the two filoviral genera, <i>Ebolavirus</i> (EBOV) and <i>Marburgvirus</i> (MARV), continue to cause episodic outbreaks in Africa. In addition, because these viruses can be aerosolized, there is concern that these viruses will be weaponized, leading to the listing of filoviruses on the CDC and NIAID select agents' Category A biodefense pathogens list. No antivirals or vaccines against these deadly hemorrhagic viruses are currently available, although a number of experimental vaccines have proven successful against homologous challenge in rodents and non-human primates. All vaccines that are being tested include in their composition the heavily glycosylated filovirus glycoprotein (GP) that is sufficient for protection against homologous lethal challenge. Evidence indicates that protection is primarily mediated by antibodies (Abs) raised against the GP that can neutralize the homologous antigen, but have little to no neutralizing capabilities against GPs from other filovirus species. The current challenge in the field is to develop an EBOV vaccine that not only protects against challenge with the homologous virus, but is also protective against other filoviruses and no univalent immunogen currently provides such broad efficacy. Studies with other enveloped viruses demonstrate that deglycosylation of the viral glycoprotein yields an immunogen that provides better protection against lethal challenge with the homologous strain of virus and yields enhanced antibody binding of more distantly related viruses. In this application, we propose to determine if removal of glycans on EBOV GP will expose cryptic epitopes within conserved regions of the glycoprotein, thereby enhancing the production of antibodies that will protect against homologous and heterologous challenge. With strong preliminary data supporting this work, we propose to assess the protection afforded by our glycan mutant GPs against EBOV and MARV challenge. This novel approach to a univalent vaccine against all filoviruses potentially solves a major ongoing challenge in the field of filovirus vaccinology.</p>			
15. SUBJECT TERMS Vaccine development, filovirus, Ebola virus, glycosylation			
16. SECURITY CLASSIFICATION OF:		17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES
			19a. NAME OF RESPONSIBLE PERSON USAMRMC

<b>a. REPORT</b>	<b>b. ABSTRACT</b>	<b>c. THIS PAGE</b>			<b>19b. TELEPHONE NUMBER</b> <i>(include area code)</i>
Unclassified	Unclassified	Unclassified	Unclassified	37	

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## 1. Introduction

The filoviruses Ebola virus (EBOV) and Marburg virus (MARV) continue to cause episodic devastating outbreaks in human and non-human primate (NHP) populations in Africa. In addition, because these viruses can be aerosolized, there is concern that these viruses will be weaponized. These characteristics have led to listing of filoviruses on the CDC and NIAID select agents' Category A biodefense pathogens lists. There are five species within the *Ebolavirus* genus, whereas all MARV strains identified to date are clustered in a single species.

EBOV (formerly Zaire ebolavirus) and Sudan (SUDV) appear to be the most virulent Ebola virus species in humans with 40-90% associated mortality. Other species include Tai forest (TAFV) (formerly Ivory Coast ebolavirus), Bundibugyo (BDBV) and Reston (RESTV). RESTV is found in bats and pigs in Asia and is associated with little to no pathology in humans.

Filovirus glycoprotein (GP) is a viral class I glycoprotein that forms a trimer of GP1/GP2 heterodimers. Mature GP1 is composed of four domains: the base, the receptor binding domain (RBD), the glycan cap and the mucin-like domain (MLD). The base and RBD are highly conserved and surrounded by a cloud of glycans that are thought to protect those critical domains from immune responses[6]. The latter two domains are heavily glycosylated with the glycan cap containing conserved N-linked glycans and the mucin-like domain (MLD) containing extensive and more variable N- and O-linked glycans.

No antivirals or vaccines against these deadly hemorrhagic viruses are currently available, although a number of experimental vaccines have proven successful against homologous challenge in rodents and NHPs. All of the efficacious vaccines include in their composition the heavily glycosylated filovirus glycoprotein (GP) and GP is sufficient in a variety of different vaccine platforms for protection against homologous lethal challenge. Evidence suggests that protection is primarily mediated by antibodies (Abs) raised against the GP that robustly neutralize the homologous antigen, but have poor to no neutralizing capabilities against GPs from heterologous species. Clearly, it is highly desirable to develop an EBOV vaccine that is cross protective against a range of Ebola virus species and MARV. No monovalent immunogen tested to date has proven to have such broad efficacy.

We proposed to develop and test a novel monovalent vaccine approach that may provide both better protection against homologous lethal challenge and broaden the cross protection against other filoviruses. **We hypothesized that targeted removal of EBOV GP1 N-linked glycans will eliminate the glycan shield surrounding the highly conserved EBOV RBD. Denuding GP1 of N-linked glycans is likely make available RBD epitopes that can generate more effective and broader immune response.** Limited studies on deglycosylation of the EBOV GP MLD suggests that loss of the EBOV GP sugar cloud will also yield enhanced immunogenicity of this viral glycoprotein. Removal of two glycans surrounding a known protective epitope in the hypervariable EBOV GP MLD resulted in increased protection in mice against homologous challenge. In a similar manner, loss of a single glycan in GP2 also increased protection. Predictions from influenza deglycosylation vaccine studies would suggest that removal of multiple glycans shielding the more highly conserved RBD and glycan cap regions would not only enhance protection against homologous challenge, but lead to better cross protection of EBOV species and perhaps of MARV as well.

**2. Keywords:** Vaccine development, filovirus, Ebola virus, glycosylation

## 3. Accomplishments:

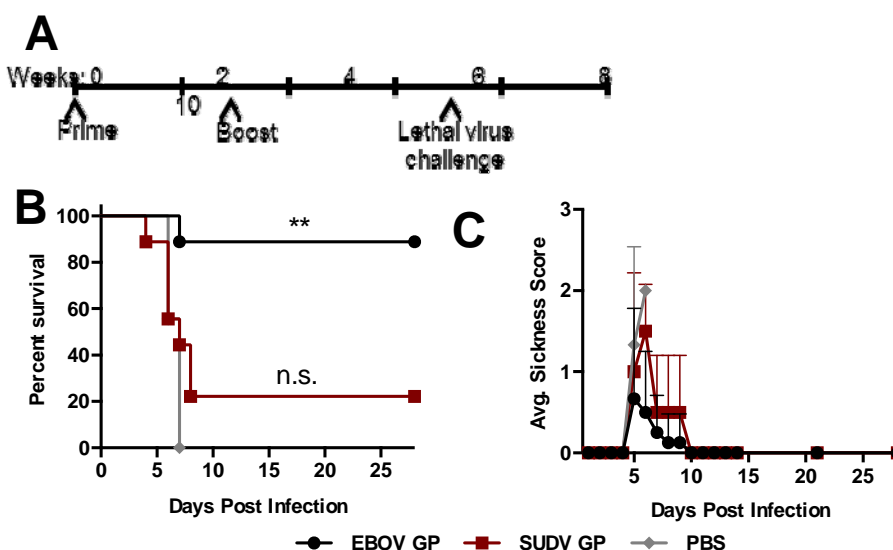
*Goals of project:*

1. Determine if EBOV GP pseudotyped VSV pseudovirions provide effective protection when delivered as a vaccine candidate.
2. Determine if targeted deglycosylation of EBOV GP1 enhances antigen immunogenicity
3. Determine if targeted deglycosylation of EBOV GP1 enhances cross protection against related ebolavirus species

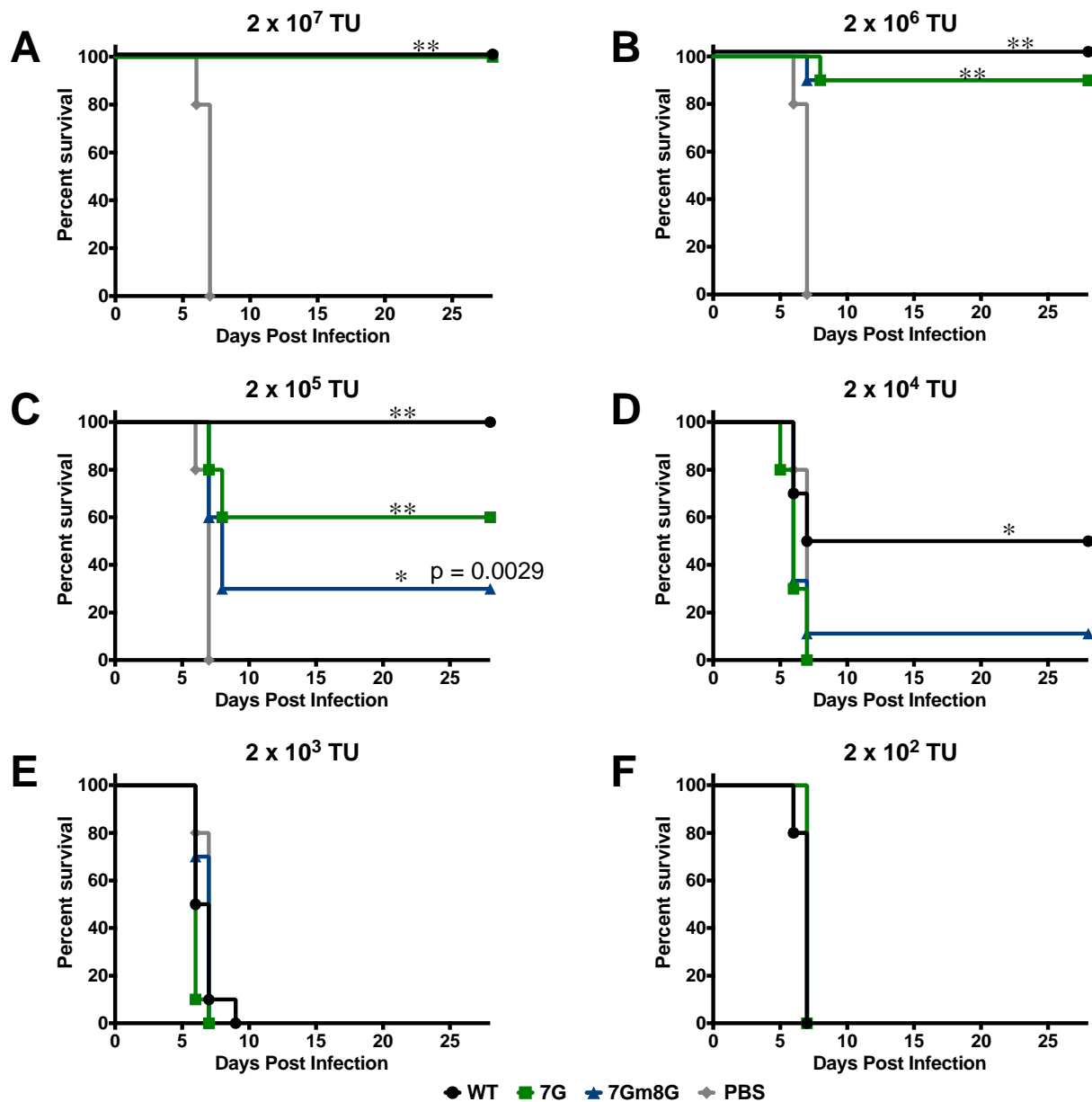
*Accomplished goals:*

All targeted objectives were tested and results are listed below:

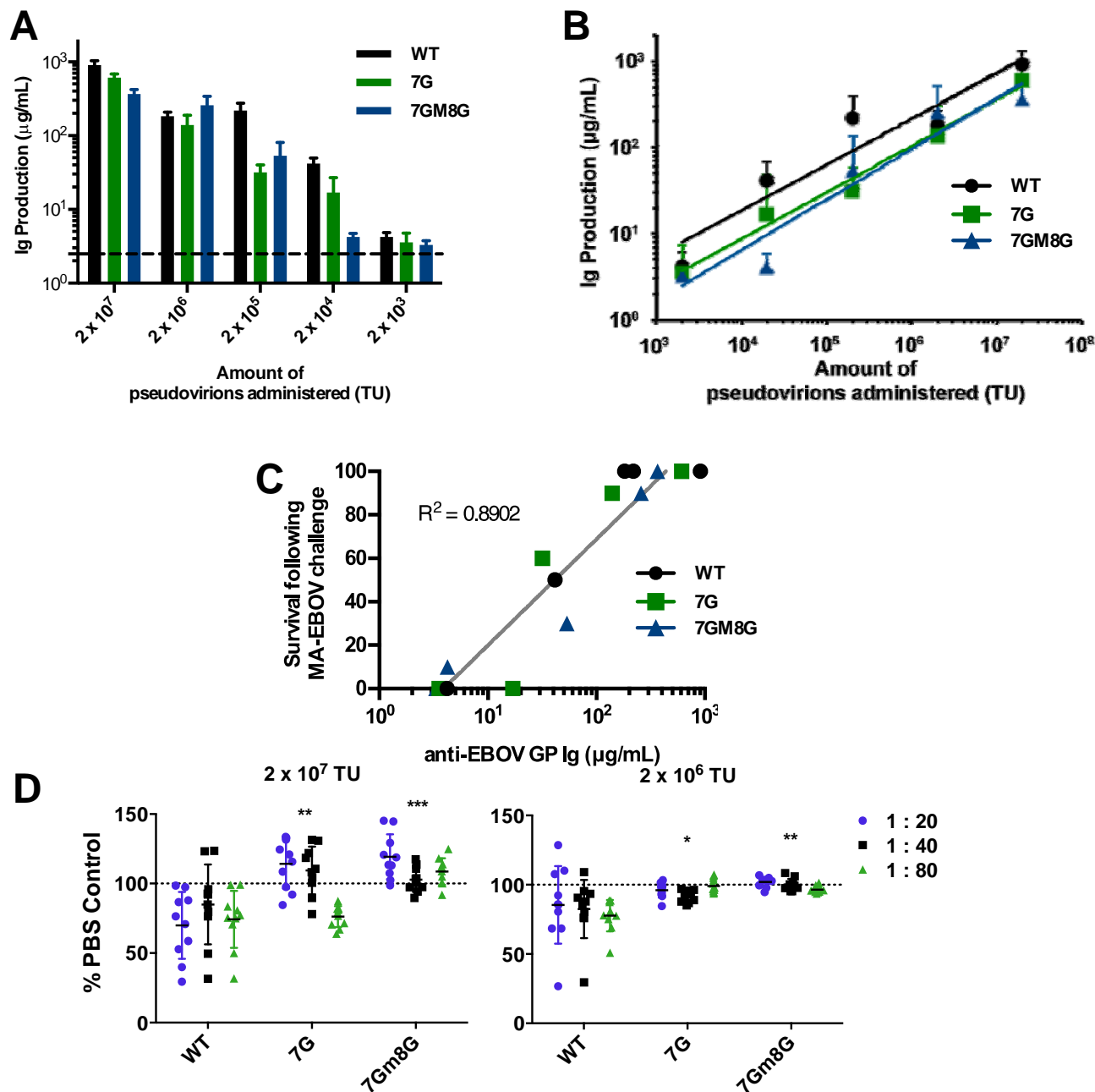
1. Vesicular stomatitis virus (VSV)-based pseudovirions bearing Ebola virus glycoprotein when delivered in a prime/boost vaccination strategy serve as highly effective immunogens (Figures 1 and 2). A prime/boost immunization provides 100% protection to mice against lethal Ebola virus challenge when  $2 \times 10^5$  to  $2 \times 10^7$  viral particles are administered as a prime/boost regime. Lower concentrations of particles ( $2 \times 10^4$ ) give partial protection.
2. Immunity conferred by our pseudovirions is dose dependent (Figure 2), correlating not with the production of neutralizing antibodies (Figure 3d), but with total immunoglobulin levels elicited (Figure 3a and b). This finding identifies a strong immune correlate for protection against lethal Ebola virus challenge which can be used in future vaccine studies.
3. Protection conferred by partially or full-deglycosylated EBOV glycoprotein on the surface of VSV pseudovirions is not as effective as wild-type Ebola virus glycoprotein (Figures 2-3). This observation is even more pronounced when lower concentrations of pseudovirions are administered as a vaccine.
4. Pseudovirions bearing Ebola virus glycoprotein with reduced numbers of N-linked glycans also does not provide effective cross protection against the distantly related *Ebolavirus*, Sudan virus (Figure 4).



**Figure 1:** Vaccination/challenge regimen demonstrating protection against homotypic challenge. (A) Vaccination regimen. (B) Protection conferred by  $2 \times 10^7$  TU of EBOV GP/VSV pseudovirions ( $n = 9/\text{group}$ ) upon challenge of BALB/c mice with 1000 PFU of MA-EBOV. Significance was determined by Mantel-Cox Test, \*\*  $p < 0.001$ . (C) Sickness score of surviving mice at indicated days post infection. Data are presented as average  $\pm$  SD.

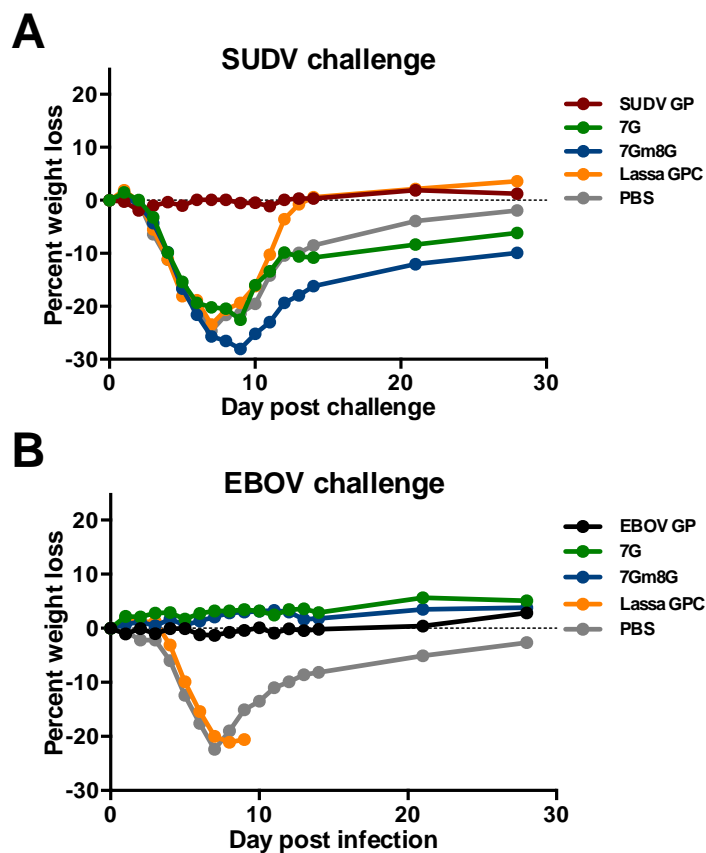


**Figure 2:** Survival of C57bl/6 females vaccinated with the indicated transducing units (TU) of VSV lacking its native glycoprotein and pseudotyped with EBOV GP (black lines), 7G (green lines), or 7Gm8G (blue lines) ( $n = 10/\text{treatment}$ ). Transducing units (TU) were determined by Vero cell transduction of VSV/EBOV GP; pseudovirion equivalent of mutant viruses were normalized to WT levels by the quantity of VSV matrix in each stock. Vaccinated mice were challenged with 100 pfu MA-EBOV and monitored for 28 days. For comparison, the PBS control is shown for each dosage. Survival of PBS control mice was compared to the other vaccine treatments with significance determined by Mantel-Cox Test, \* $p < 0.01$ , \*\* $p < 0.001$ .



**Figure 3:** Humoral responses in mice vaccinated with WT EBOV GP/VSV were more robust than that of mice vaccinated with deglycosylated pseudovirions. Sera were collected from vaccinated mice three weeks following boost. (A) Anti-EBOV GP Ig levels. Dashed line represents Ig levels observed in PBS-treated mice. (B) Correlation between the quantity of pseudovirus administered as a vaccine and Ig production. Trend line for each vaccine treatment is shown independently. (C) Correlation between group survival and anti-EBOV GP Ig in sera. Trend line is shown for pooled results from all three pseudovirion treatments. (D) Neutralizing activity present in vaccinated mice sera. Serum was serially diluted and incubated with EBOV GP/VSV for 30 minutes at 37°C prior to addition to Vero cells. Data are shown as transduction (GFP positive cells) presented as a percentage of PBS control. Significant differences in neutralization conferred by EBOV GP compared to either of the deglycosylated EBOV GP groups were determined by two-way ANOVA with Tukey post hoc analysis, \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0001$ .





**Figure 4:** VSV pseudovirions bearing deglycosylated EBOV GP do not provide protection against SUDV challenge. C57bl/6 IFNAR<sup>-/-</sup> (n=10 mice) were primed and boosted with  $2 \times 10^7$  TU of the indicated VSV pseudovirions followed by challenge with (A) 1000 pfu of WT SUDV or (B) 1000 pfu of WT EBOV. Percentage of weight loss is shown at the indicated time following infection.

#### *Training and professional development:*

While this project was not designed to provide professional training and development, both a graduate student and post-doctoral fellow worked on this project, resulting in training. Bethany Rhein worked on this as part of her Ph.D. work. She is included as a co-author on the manuscript. In addition, Rachel Brouillette also worked on this project as a portion of her Ph.D. work. Ms. Brouillette gave an oral presentation on the study in November, 2015 at the Vaccines Against Antigenically Variable Viruses Symposium in Ames, Iowa. Dr. Natarajan Ayithan was also worked on this study. All trainees worked with Dr. Maury directly on the project obtaining increased knowledge and skill in the area of vaccine development.

#### *Dissemination of results:*

1. Manuscript on this work was submitted to Journal of Virology (JVI) in Fall, 2015. We are currently working on requested alterations/additions to the manuscript and anticipate resubmission shortly. A pdf of the manuscript is attached as an appendix to this document
2. An oral presentation of the work was given at the Vaccines Against Antigenically Variable Viruses Symposium in Ames, Iowa in November, 2015.

#### **4. Impact:**

##### *Impact on development of principal discipline:*

We have made a number of important observations. We have shown that a VSV pseudovirion bearing Ebola virus glycoprotein on its surface serves as a highly immunogenic vaccine. Further, we have demonstrated that removal of some or all of the GP1 N linked glycans reduces immunogenicity of the antigen rather than enhancing immunogenicity. We have also shown that the deglycosylated vaccine candidates do not provide good cross protection against other ebolavirus species. Finally, we demonstrate that vaccine protection did not correlate with production of neutralizing antibodies, but did correlate well with the overall production of Ebola virus glycoprotein specific immunoglobulins.

##### *Impact on other disciplines:*

This body of work provides vaccine delivery correlates for not only filoviruses but for the development of other viral (and perhaps bacterial) vaccines.

##### *Impact on technology transfer:*

Nothing to report

##### *Impact on society:*

Development of a safe, highly efficacious vaccine candidate for Ebola virus has profound implications for society, particularly in Africa.

## 5. Changes/ Problems:

None to report. Study was completed as proposed.

## 6. Products:

Journal publication: Manuscript on this work was submitted to Journal of Virology (JVI) in Fall, 2015. We are currently working on requested reviewer alterations/additions to the manuscript and anticipate resubmission shortly. A pdf of the manuscript is attached as an appendix to this document.

Meeting presentation: An oral presentation of the work was given at the Vaccines Against Antigenically Variable Viruses Symposium in Ames, Iowa in November, 2015.

*Website:*

None to report.

*Technologies:*

We have developed a new and safer vaccine platform for filoviruses.

*Patent applications:*

We have submitted an invention disclosure to University of Iowa Foundation for this vaccine platform. The information is currently being considered by the Foundation for a patent application.

*Other products:*

None to report.

## 7. Participants:

Name:	<i>Wendy Maury</i>
Project Role:	<i>Principal Investigator</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	2.5
Contribution to Project:	<i>Dr. Maury oversaw the entire project. In addition, she was responsible for most of the work performed, including production and characterization of pseudovirions, administration of vaccine, interaction with USAMRIID individuals, collation of data and writing of the manuscript.</i>
Funding Support:	.

Name:	<i>Bethany Rhein</i>
Project Role:	<i>Graduate Student</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Ms. Rhein worked with mice and mouse sera assessing the immunoglobulin levels produced by our vaccine.</i>
Funding Support:	<i>NIH RO1 funding as well as this award.</i>

Name:	<i>Rachel Brouillette</i>
Project Role:	<i>Graduate Student</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>1</i>
Contribution to Project:	<i>Ms. Brouillette worked with mice and mouse sera assessing the immunoglobulin levels produced by our vaccine.</i>
Funding Support:	<i>NIH – a training grant</i>
Name:	<i>Natarajan Ayithan</i>
Project Role:	<i>Post doctoral fellow</i>
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	<i>5</i>
Contribution to Project:	<i>Dr. Ayithan helped with mice and worked in parallel with Dr. Maury on the project.</i>
Funding Support:	<i>NIH R21 funding as well as funding from this project.</i>

*Other organizations:*

None to report.

**Vesicular stomatitis virus pseudotyped with Ebola virus glycoprotein serves as  
a highly protective, non-infectious vaccine against Ebola virus challenge**

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Running title: Pseudovirion vaccine against Ebola virus

## Abstract

An epidemic caused by Ebola virus (EBOV) continues in West Africa, demonstrating the significant public health burden of filovirus infections and highlighting the need for preventive measures to combat the associated disease. Since, no vaccines or antivirals are currently FDA approved, we sought to assess protection conferred by an EBOV vaccine composed of non-infectious vesicular stomatitis virus (VSV) pseudovirions bearing EBOV glycoprotein (GP). A prime/boost vaccination regime protected mice against lethal challenge with mouse-adapted Ebola virus (MA-EBOV) in a dose-dependent manner. As N-linked glycans are thought to shield conserved regions of GP, we also tested if pseudovirions containing GPs lacking N-linked glycans on GP1 would provide effective immunity. High doses of GP/VSV partially or fully denuded of N-linked glycans on GP1 protected mice against MA-EBOV challenge. However, deglycosylated mutants proved less effective than WT GP/VSV at lower doses. Further, neither N-linked glycan deficient GP/VSV provided significant cross protection against Sudan virus. As others have reported, serum from vaccinated mice that were protected against lethal challenge had few to no detectable neutralizing antibodies, indicating that EBOV vaccines do not need to elicit neutralizing antibodies to protect against lethal challenge. A strong correlation was found between the amount of vaccine-induced GP-specific Ig and protection. Our results show that non-infectious GP/VSV pseudovirions serve as a successful vaccination platform, but reduction of the glycan shield is not an effective means of enhancing immunogenicity of EBOV GP. Further, we identify that GP-specific Ig levels provide a good immune correlate of protection.

## 42 **Importance**

43 The current West Africa Ebola virus epidemic continues despite international efforts. While  
44 human vaccine trials are underway, no FDA-approved vaccines are yet available and it remains  
45 unclear which vaccine platform will serve as the most efficacious and safest approach. Here, we  
46 test the efficacy of a novel filovirus vaccine platform and find that wild-type Ebola virus  
47 glycoprotein, in the context of this platform, provides robust protection. Further, we investigated  
48 if removal of the heavy glycan shield surrounding the glycoprotein enhances vaccine efficacy.  
49 Surprisingly, we found that removal of glycans decreases the efficacy of the vaccine, reducing  
50 the protection offered against lethal challenge with Ebola virus.

## Introduction

Filoviruses, such as Ebola virus (EBOV) and Marburg virus (MARV), cause sporadic outbreaks of viral hemorrhagic fever throughout Central Africa. At the time of this report, a EBOV epidemic continues in West Africa, a region that has not previously experienced filovirus outbreaks (13).

A number of different vaccine platforms that express the filovirus glycoprotein (GP) have proven to be effective at protecting against lethal homotypic filovirus challenge in animal models (15, 31-35, 38, 40). This series of studies has led the field to conclude that immune responses against filovirus GPs are necessary and sufficient for protection. Vaccine platforms expressing the filoviral GP that have proved efficacious in at least one animal model include: DNA plasmids, adenoviral vectors, virus-like particles, recombinant Venezuelan equine encephalitis virus particles and infectious recombinant viruses, such as human parainfluenza virus type 3, rabies virus and vesicular stomatitis virus (VSV) (reviewed in (7, 11)). Surprisingly, EBOV GP pseudovirions have not been assessed for their efficacy as a vaccine platform. EBOV GP pseudotyped on to vesicular stomatitis virus (VSV) has many advantages as a vaccine platform, including ease of production and characterization, absence of virus replication concerns and the robust immune stimulatory activity associated with VSV proteins (5).

The highly effective EBOV immunogen, GP, is produced from a pro-protein that is processed by furin in cells to produce GP1/GP2 heterodimers. These class I heterodimeric glycoproteins reside as trimers on the surface of infected cells and virions. Mature GP1 sits on top GP2 which is anchored in the membrane. GP1 contains four different domains, including a base, receptor-binding domain (RBD), glycan cap and mucin-like domain (MLD). The first three domains compose the core of GP1 and are required for expression and function of the pre-fusion



glycoprotein, whereas the MLD is not required for virion entry or GP expression (14). GP1 is extensively glycosylated with approximately half of the mature GP mass contributed by N- and O-linked glycans (14). Fifteen N-linked glycans are found on EBOV GP1 and as many as 80 O-linked glycans are thought to be present on the MLD (14, 21, 22). The transmembrane protein GP2 contains two N-linked glycans on its ectodomain that are conserved throughout the entire virus family (14, 21, 23).

Glycans on viral glycoproteins have been shown to facilitate immune evasion through shielding the protein from neutralizing antibodies (1, 2, 8, 19, 39, 41). For example, antibodies raised against influenza A virus hemagglutinin (HA), bearing truncated glycans, have enhanced antigen binding and neutralization of virus. Furthermore, decreasing the complexity of N-linked glycans on HA increased the efficacy of a HA subunit vaccine in mice (39). Previous work with EBOV indicated that vaccination with virus-like particles (VLPs) expressing EBOV GP lacking the MLD resulted in up to 2.5-fold higher neutralizing antibody titers compared to wild-type (WT) GP on VLPs (25), suggesting glycan shielding compromises EBOV GP immunogenicity. Occlusion of the GP1 RBD is thought to be conferred by N-linked glycans present on the core of GP1 and N- and O-linked glycans on the MLD as shown in Figure 1A (left panel). Consistent with the possibility that glycan shielding might reduce immune responses to EBOV GP, we have previously shown that removal of all N-linked glycans from EBOV GP1 increases convalescent antibody-dependent neutralization without compromising GP incorporation into VSV pseudovirions (22). By modeling, complete elimination of N-linked glycans on GP1 as is found on our GP mutant 7Gm8G would be predicted to result in greater surface availability of the highly conserved GP1 RBD (Figure 1A, right panel). In contrast to these studies, a previous study by Dowling, et al. hinted that loss of glycans might decrease the immune-stimulating

efficiency of EBOV GP. They demonstrated that protection offered by GP was diminished by deleting the MLD or mutation of one of the two GP2 N-glycans (6).

Here, we evaluate the efficacy of EBOV GP pseudotyped onto VSV as a vaccine candidate and find robust protection conferred against lethal challenge with mouse-adapted EBOV (MA-EBOV). Protection strongly associated with production of EBOV GP-specific immunoglobulins. We also assessed if removal of N-linked glycans from EBOV GP1 leads to increased immunogenicity and improved vaccine efficacy by exposing epitopes that are masked in the RBD of the WT GP. Further, we evaluated if these deglycosylated GPs provide better cross protection against other *Ebolavirus* members, such as Sudan virus (SUDV). We demonstrate that these N-linked glycan site (NGS) mutants provide poorer protection against MA-EBOV challenge and offer little to no protection against SUDV challenge.

## **Materials and Methods**

**Cell lines and plasmids.** Vero cells and HEK293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The pcDNA3.1 expression plasmids for EBOV GP (accession number: NP\_066246) and the N-linked glycan-deficient GP1 mutants 7G and 7Gm8G have been previously described (22). Codon optimized SUDV-Boniface GP (a gift from Robert Davey, accession number: Q66814) containing a Q95K mutation, which we have determined increases pseudovirion titer (data not shown), was expressed from pcDNA3.1. The pCAGGS vector was used to express codon optimized Lassa virus - Josiah (LASV) glycoprotein (accession number: NP\_694870).

**Modeling of GP N-linked glycans.** The addition of complex N-linked glycans to the pre-fusion EBOV GP<sub>1,2</sub> ΔTM structure (PDB ID: 3CSY) that lacks the MLD was performed as previously described (22, 23). Briefly, the published structure lacks four NGS in GP1 due to disordered regions missing from the structure (N204 and N296) or mutations that promoted crystallization (N40 and N228) (20, 21). The EBOV GP sequence was submitted to the PHYRE2 protein fold recognition server (16), which provided a structure that contained NGS at N40 and N228. This structure was submitted for *in silico* glycosylation using the GlyProt server (glycosciences.de), which produced a model containing complex N-linked glycans at all NGS, except N204 and N296, which are part of disordered regions (21). Complex glycans at these sites were modeled onto the glycosylated structure at predicted sites with PyMol.

**Production of VSV/GFP pseudovirions.** Pseudovirions were produced in HEK293T cells as previously described (17, 37). Briefly, HEK293T cells were transfected with the various viral GP-expressing constructs and at 24 h transduced by an infection defective vesicular stomatitis virus that lacks the gene encoding the native G glycoprotein (VSVΔG-GFP) pseudotyped with LASV GPC. In the genome of VSVΔG-GFP, glycoprotein G gene is replaced with the green fluorescence protein gene (GFP). After 24 h, cell supernatants were collected and filtered through 0.45 μm syringe-filters followed by storage at -80°C. Pseudovirions were concentrated overnight by centrifugation at 5400 x g. Virus was resuspended in PBS and pelleted through a 20% sucrose cushion for 2 h at 83,000 x g and resuspended in PBS, treated with Detoxi-Gel endotoxin removing resin (Thermo Scientific) to remove any endotoxin in our preparations. All virus stocks were frozen at -80°C until use.

**VSV-matrix quantification to normalize pseudovirion administration.** Assessment of VSV-matrix (M) in pseudovirion preparations was performed as previously described (22). Briefly,

pseudovirion stocks were passed through a dot blot apparatus onto nitrocellulose. VSV-M was detected with mouse anti-VSV-M mAb 23H12 (24). Signals were quantified using the Odyssey Imaging Station and Image Studio software (LI-COR).

**Transduction assays.** Vero cells were seeded in 48-well plates 24 h prior to transduction. Serial dilutions of WT EBOV GP/VSV pseudovirion stocks were added to Vero cell monolayers. Transduction was determined by quantification of GFP expressing cells by flow cytometry 24 h following addition of pseudovirions.

**Ethics statement.** Animal research at the University of Iowa was conducted under a protocol approved by the University of Iowa Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. Animal research was conducted under a protocol approved by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) Institutional Animal Care and Use Committee (IACUC) in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. Both facilities are fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International and adhere to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

Challenge studies were conducted under maximum containment in the USAMRIID animal biosafety level 4 (ABSL-4) facility.

**Vaccinations and EBOV challenges.** The amount of the different pseudovirions administered in each vaccine study was based on equivalent matrix levels to that of EBOV GP/VSV. In all challenge studies using mouse-adapted EBOV, mice were challenged with greater than 3000

lethal dose<sub>50</sub> (LD<sub>50</sub>) of mouse-adapted Ebola virus (MA-EBOV) intraperitoneally (i.p.). Depending on the stock used, a dose of 3000 LD<sub>50</sub> ranged from 100 to 1000 pfu.

*Experiment 1:* 4-8 week old BALB/c males and females (n=9/group) were administered  $2 \times 10^7$  WT EBOV GP/VSV transducing units (TU) in PBS intramuscularly (i.m.) as a prime and then a boost 3 weeks later. The quantity of SUDV GP/VSV pseudovirions administered was based on matrix equivalents to that of EBOV GP/VSV using the same prime/boost strategy. Vaccinated mice were shipped to USAMRIID (Fort Detrick, MD) where at 7 weeks after initiation of the vaccination regime they were challenged with greater than 3000 lethal dose<sub>50</sub> (LD<sub>50</sub>) of mouse-adapted Ebola virus (MA-EBOV) intraperitoneally (i.p.).

*Experiment 2:* 6 week old C57bl/6 females were purchased from Charles River. Ten-fold serial dilutions in PBS of WT EBOV GP/VSV pseudovirions ( $2 \times 10^2$  to  $2 \times 10^7$  TU) were given i.m. as a prime and then a boost 3 weeks later (n=10/group). The quantity of other VSV pseudovirions (7G and 7Gm8G) administered was based on matrix equivalents to that of EBOV GP/VSV using the same dilution series and prime/boost strategy. Vaccinated mice were shipped to USAMRIID (Fort Detrick, MD) where at seven weeks following initiation of the vaccination regime, mice were challenged with greater than 3000 lethal dose<sub>50</sub> (LD<sub>50</sub>) of mouse-adapted Ebola virus (MA-EBOV) intraperitoneally (i.p.). Challenged mice were observed daily for lethality or clinical signs of disease, including, but not limited to, reduced grooming, hypo-activity and weight loss. Daily observations were increased to a minimum of twice daily while mice were exhibiting signs of disease. Moribund mice were humanely euthanized based on IACUC-approved criteria

*Experiment 3:* 4- 8 week old male and female mice lacking the interferon alpha/beta receptor (C57bl/6.129S2-Ifnar1tm1Agt/Mmjax; referred to in this publication as IFNAR<sup>-/-</sup>) were purchased from Jackson Laboratories (Bar Harbor, ME) and used for all SUDV challenge

experiments (n= 10/group). Upon arrival, mice were housed in microisolater cages and provided chow and water *ad libitum*. All vaccinated mice received the matrix equivalents of  $2 \times 10^7$  transducing units of WT EBOV GP/VSV i.m. in a prime/boost regime. Seven weeks following vaccinations, mice were challenged i.p. with 1000 pfu of SUDV (Boniface isolate) or 1000 pfu of WT EBOV. Mice were monitored daily as described above.

**IgG titers and neutralization assays.** Serum was taken from mice in our second vaccination study (serial dilution study) and assessed for immunoglobulin detecting the core of WT EBOV GP by ELISA. Briefly,  $2 \times 10^5$  TU of purified EBOV GPΔMLD/FIV pseudovirions (as assessed on SNB-19 cells) were coated overnight in PBS at 4°C in an Immulon HB 96-well plate. FIV pseudovirions were generated as previously described (12). Virus was diluted in PBS. The standard curve was composed of a two-fold dilution series of mouse Ig fraction (ImmunoReagent, Inc). Plates were blocked for 1h at room temperature with PBS with 2% bovine serum albumin (BSA), incubated with serial dilutions (1:1000, 1:10,000 and 1:1000,000) of serum for overnight at 4°C, probed with 1 µg/ml HRP-conjugated rabbit anti-mouse (Pierce) for 1 h and wells were assessed for HRP activity with UltraTMB (Thermo). Absorbance was read at 405 nm.

To assess the ability of our vaccinated mouse serum to neutralize EBOV GP transduction, serial dilutions of serum were incubated with WT EBOV GP VSV pseudovirions (WT MOI ~0.3), for 30 min at 37 °C. Reactions were then diluted 5-fold in media and used to transduce confluent monolayers of Vero cells. Inhibition curves and half maximal inhibitory concentrations (IC<sub>50</sub>) were determined using GraphPad Prism 5.

## Results

Vaccination regimen and challenge timing are shown in Figure 1B. In initial studies, we determined if EBOV GP/VSV pseudovirions protect against MA-EBOV challenge. Mice were primed i.m. with  $2 \times 10^7$  TU of VSV lacking the native G glycoprotein gene and pseudotyped with EBOV GP or SUDV GP. Three weeks later, the same quantity of viruses was re-administered to the mice as a boost. A PBS injection served as the control. Mice were challenged i.p. with 1000 PFU of mouse-adapted Ebola virus (MA-EBOV) approximately 7 weeks after the prime. Challenged mice were monitored for 28 days post infection (dpi) (Figure 1C). While none of the PBS group survived, 8 out of 9 EBOV GP/VSV vaccinated mice were protected from challenge. In contrast, SUDV GP/VSV-vaccinated mice were poorly protected against MA-EBOV challenge. Consistent with the survival findings, GP/VSV vaccinated mice trended towards lower average sickness scores than the PBS controls or the SUDV/VSV-vaccinated mice (Figure 1D). The poor cross protection provided by SUDV GP/VSV against MA-EBOV challenge was not unexpected as the lack of sufficient cross protection against other *Ebolavirus* species has been previously reported (15, 26). These studies provided an initial indication that our VSV pseudovirions bearing EBOV GP offers effective protection against homotypic challenge.

In a second study, we assessed the efficacy of our vaccine platform over a range of concentrations of viral particles containing WT EBOV GP. Additionally, we evaluated if our partially or fully deglycosylated pseudovirions conferred similar protection to that provided by our WT virions. In this study, 6 week old C57bl/6 females were given a prime/boost regimen of  $2 \times 10^2$  to  $2 \times 10^7$  TU of EBOV GP/VSV, 7G/VSV or 7Gm8G/VSV. Vaccinated mice were challenged with 100 pfu of MA-EBOV at seven weeks following the initial prime and monitored

for 28 dpi. As expected, mice given PBS succumbed to disease 6-7 dpi (Figure 2). The two highest doses of all three EBOV GP pseudovirion vaccines protected against lethal MA-EBOV challenge (Figure 2A and B).

Lower doses of VSV pseudovirions bearing 7G, or 7Gm8G proved to be less effective than EBOV GP/VSV. The reduced efficacy of our deglycosylated GP pseudovirions became evident at prime/boosts composed of  $2 \times 10^5$  TU or less (Figure 2C-F), indicating that mutation of NGS within GP1 decreased the immunogenicity of GP rather than improving it.

To determine if exposure of highly conserved regions of GP1 through deglycosylation led to quantitative changes in anti-EBOV GP antibodies, we assessed the amount of immunoglobulin (Ig) in convalescent serum from each vaccination group that bound to EBOV GPΔMLD pseudotyped on to feline immunodeficiency virus (Figure 3A). A MLD-deleted EBOV GP was selected as the target in these ELISAs since recent findings indicate that antibodies against the core of EBOV GP are sufficient for protection (29). The quantity of anti-EBOV GP Ig produced by the different vaccination treatments correlated strongly with dose of immunogen administered (Figure 3A and B). High levels of Ig were detected with doses of either  $2 \times 10^6$  or  $2 \times 10^7$  TU, independent of the glycosylation status of the GP. However, with administration of intermediate concentrations of pseudovirions, more anti-EBOV GP antibodies were detected in the EBOV GP pseudovirions group than either partially deglycosylated (7G) or fully deglycosylated (7Gm8G) as evidenced by the trend line (Figure 3B). A strong, positive correlation between the amount of anti-EBOV GP Ig and the percentage of survival for each group was also evident (Figure 3C), suggesting that anti-EBOV GP Ig production might serve as a good immune correlate for vaccine protection.



Given the positive association of serum neutralizing antibodies with survival against EBOV in human and animal model infections (18, 36, 40) and our observed correlation between survival and anti-EBOV GP antibody levels in convalescent serum, we evaluated convalescent sera from vaccinated mice for the ability to neutralize transduction by EBOV GP/VSV pseudovirions. Sera from mice vaccinated with either  $2 \times 10^7$  or  $2 \times 10^6$  TU of WT EBOV GP/VSV contained modest levels of neutralizing antibodies (Figure 3D). Neither GP mutant elicited detectable neutralizing antibody responses even at the highest concentrations of immunogen, despite providing robust protection against lethal challenge. These results indicate that VSV pseudovirion-based vaccines against EBOV elicit a protective immune response independent of neutralizing antibodies. Interestingly, protection conferred by other EBOV vaccines is not always reported to be strongly correlated with the production of high levels of neutralizing antibodies (26, 30).

Finally, we evaluated the possibility that our EBOV GP immunogens lacking N-linked glycans could provide cross protection against the distantly related ebolavirus, SUDV-Boniface. A similar vaccination schedule was used in this study, injecting a prime/boost of  $2 \times 10^7$  7G or 7Gm8G pseudovirions into C57bl/6 mice lacking the interferon  $\alpha/\beta$  receptor (IFNAR<sup>-/-</sup>). IFNAR<sup>-/-</sup> mice were used in these experiments as a mouse-adapted strain of SUDV that is lethal to immunocompetent mice is not currently available and previous work has demonstrated that IFNAR<sup>-/-</sup> mice have significant and consistent weight loss upon WT SUDV infection (10). WT EBOV GP pseudovirions were not evaluated for their ability to confer protection against SUDV as others have previously demonstrated that WT EBOV GP does not provide cross protection (10). Mice vaccinated with PBS, SUDV GP/VSV, 7G GP/VSV or 7Gm8G/VSV pseudovirions were challenged with 1000 PFU of WT SUDV(Boniface) i.p. and mice were weighed daily as

previously described (3, 4). While SUDV GP/VSV pseudovirions protected against weight loss, 7G nor 7Gm8G had weight loss similar to that of pseudovirions bearing Lassa virus GPC or the PBS control, indicating our deglycosylated mutant EBOV GPs were unable to provide protection, despite that the same vaccination dosage protected well against WT EBOV challenge (Figure 4A and B).

## Discussion

In the present study, we sought to determine if a prime/boost vaccination of mice with non-infectious VSV pseudotyped with EBOV GP provides protection from lethal EBOV challenge. We found that prime/boost administration of  $2 \times 10^5$  TU or greater of EBOV GP/VSV proved efficacious. Protection was observed in both BALB/c and C57bl/6 mice, indicating that the vaccine was effective independent of the strain of mouse used. These findings provide evidence that VSV pseudovirion-based vaccines may provide a highly effective and safe alternative to the infectious recombinant VSV platform that is currently in human trials.

Through our dose response studies, we have identified that total anti-EBOV GP Ig levels serve as an excellent immune marker predictive for protection. Not surprisingly, the amount of EBOV GP-specific Ig that was detected was strongly correlated with the dosage of pseudovirions administered. Regardless of the glycosylation status of the virions, doses of  $2 \times 10^6$  TU or higher resulted in anti-EBOV GP Ig levels of 135  $\mu$ g/ml or higher in our assay and protected 90 to 100% of mice from lethal MA-EBOV challenge. Vaccine doses that resulted in production of 30 to 60  $\mu$ g/ml of EBOV GP-specific Ig provided 30 to 60% protection and doses producing Ig levels similar to that of the PBS control provided no significant protection. Interestingly, this robust correlation with Ig responses was not associated with detectable neutralization of EBOV

GP/VSV pseudovirions in our in vitro assays. In combination, our findings suggest that our vaccine elicits protective humoral responses that do not involve neutralization of viral particle in in vitro assays.

We also explored if increased protection was conferred by pseudovirions containing EBOV GP to pseudovirions containing mutant EBOV GPs lacking N-linked glycans on GP1. Interestingly, decreasing the glycan shield on EBOV GP diminished the ability of moderate vaccine doses to protect against MA-EBOV, indicating that loss of EBOV GP1 NGS is not an effective strategy to increase immunogenicity of EBOV GP. As we have shown that elimination of GP1 NGS increases the sensitivity of GP to endosomal proteolytic processing (22), it is possible that removal of N-linked glycans decreases the stability and antigen presentation of GP *in vivo*, resulting in poor immune stimulation at lower doses.

These studies also demonstrate that pseudovirions bearing NGS-denuded GP1 provide poor cross protection against SUDV challenge. Thus, our studies provide no evidence that an EBOV GP immunogen lacking NGS on GP1 elicits novel protective immune responses to highly conserved regions of GP. To date, the most effective cross-protection has been achieved through combinatorial vaccines that present proteins from multiple viral species to the host (9, 10, 26-28, 31). Together with our results, a mixture of VSV pseudotyped with representative GPs from the filovirus family could provide an efficient and safe vaccine that warrants further investigation in other animal model systems.

## Acknowledgements

Opinions, conclusions, interpretations, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. The mention of trade names or commercial products does not constitute endorsement or recommendation for use by the Department of the Army or the Department of Defense. This work was supported by funding from the National Institutes of Health (R01 AI077519 to WM, T32AI007533 to support NL, T32 AI996343 to support CMH) and Department of Defense (CB3947 to JD).

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## Figure legends

### **Figure 1:** Vaccination/challenge regimen demonstrating protection against homotypic challenge.

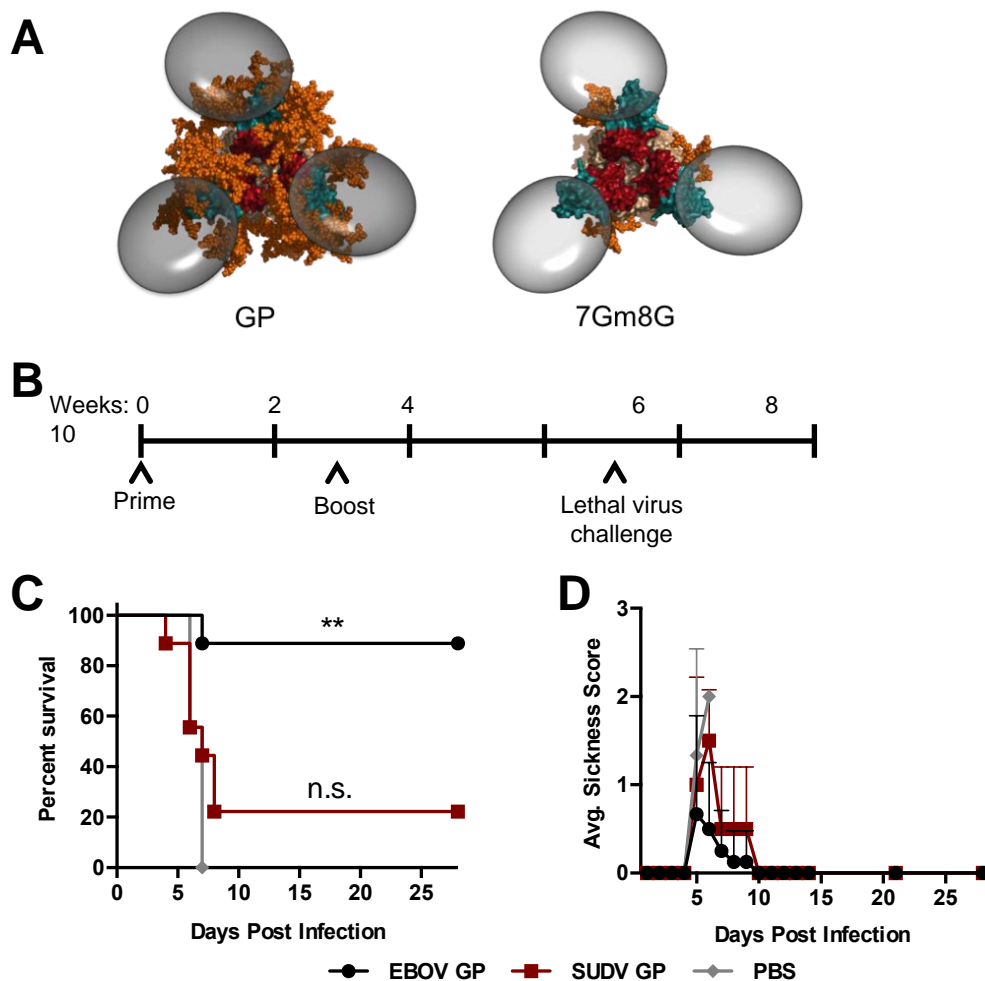
(A) Top-down view of models of EBOV GP and a GP mutant fully deglycosylated for GP1 N-linked glycans. RBD is shown in red, glycan cap is shown in teal, GP2 is shown in tan, N-linked glycans are shown in orange, and the MLD (not included in the crystal structure) is modeled as a gray sphere. Two N-linked glycans found on GP2 were not mutated in our glycosylation mutants investigated in these studies and are shown around the base of GP2. *Left*, WT GP; *right*, 7Gm8G mutant lacking all GP1 N-linked glycans indicated by the absence of orange glycans modeled on GP1 and an increase in transparency of the MLD. (B) Vaccination regimen. (C) Protection conferred by  $2 \times 10^7$  TU of EBOV GP/VSV pseudovirions ( $n = 9/\text{group}$ ) upon challenge of BALB/c mice with 1000 PFU of MA-EBOV. Significance was determined by Mantel-Cox Test,  $** p < 0.001$ . (D) Sickness score of surviving mice at indicated days post infection. Data are presented as average  $\pm$  SD.

**Figure 2:** Survival of C57bl/6 females vaccinated with the indicated transducing units (TU) of VSV lacking its native glycoprotein and pseudotyped with EBOV GP (black lines), 7G (green lines), or 7Gm8G (blue lines) ( $n = 10/\text{treatment}$ ). Transducing units (TU) were determined by Vero cell transduction of VSV/EBOV GP; pseudovirion equivalent of mutant viruses were normalized to WT levels by the quantity of VSV matrix in each stock. Vaccinated mice were challenged with 100 pfu MA-EBOV and monitored for 28 days. For comparison, the PBS control is shown for each dosage. Survival of PBS control mice was compared to the other vaccine treatments with significance determined by Mantel-Cox Test,  $*p < 0.01$ ,  $**p < 0.001$ .

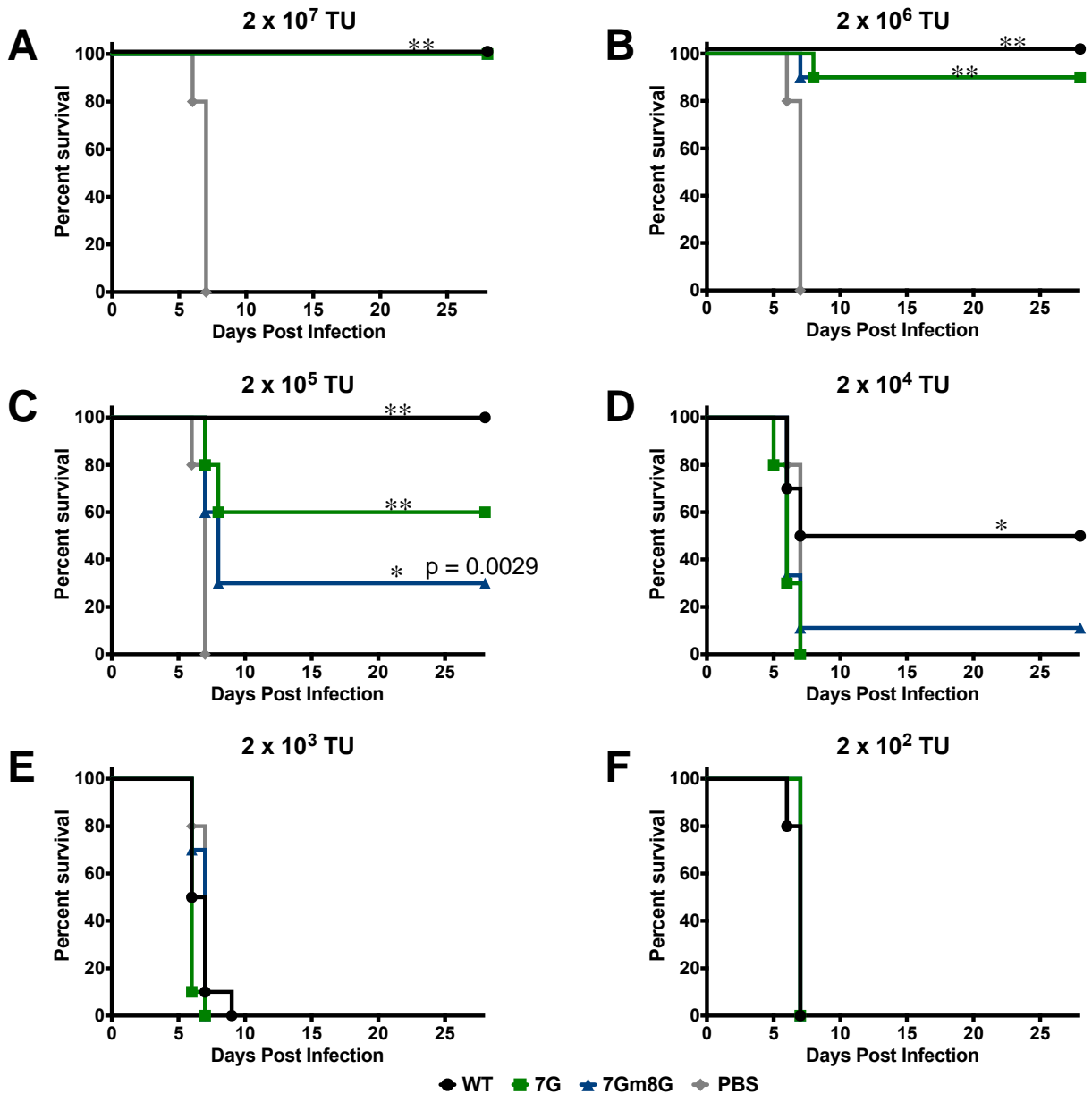


**Figure 3:** Humoral responses in mice vaccinated with WT EBOV GP/VSV were more robust than that of mice vaccinated with deglycosylated pseudovirions. Sera were collected from vaccinated mice three weeks following boost. (A) Anti-EBOV GP Ig levels. Dashed line represents Ig levels observed in PBS-treated mice. (B) Correlation between the quantity of pseudovirus administered as a vaccine and Ig production. Trend line for each vaccine treatment is shown independently. (C) Correlation between group survival and anti-EBOV GP Ig in sera. Trend line is shown for pooled results from all three pseudovirion treatments. (D) Neutralizing activity present in vaccinated mice sera. Serum was serially diluted and incubated with EBOV GP/VSV for 30 minutes at 37°C prior to addition to Vero cells. Data are shown as transduction (GFP positive cells) presented as a percentage of PBS control. Significant differences in neutralization conferred by EBOV GP compared to either of the deglycosylated EBOV GP groups were determined by two-way ANOVA with Tukey post hoc analysis, \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0001$ .

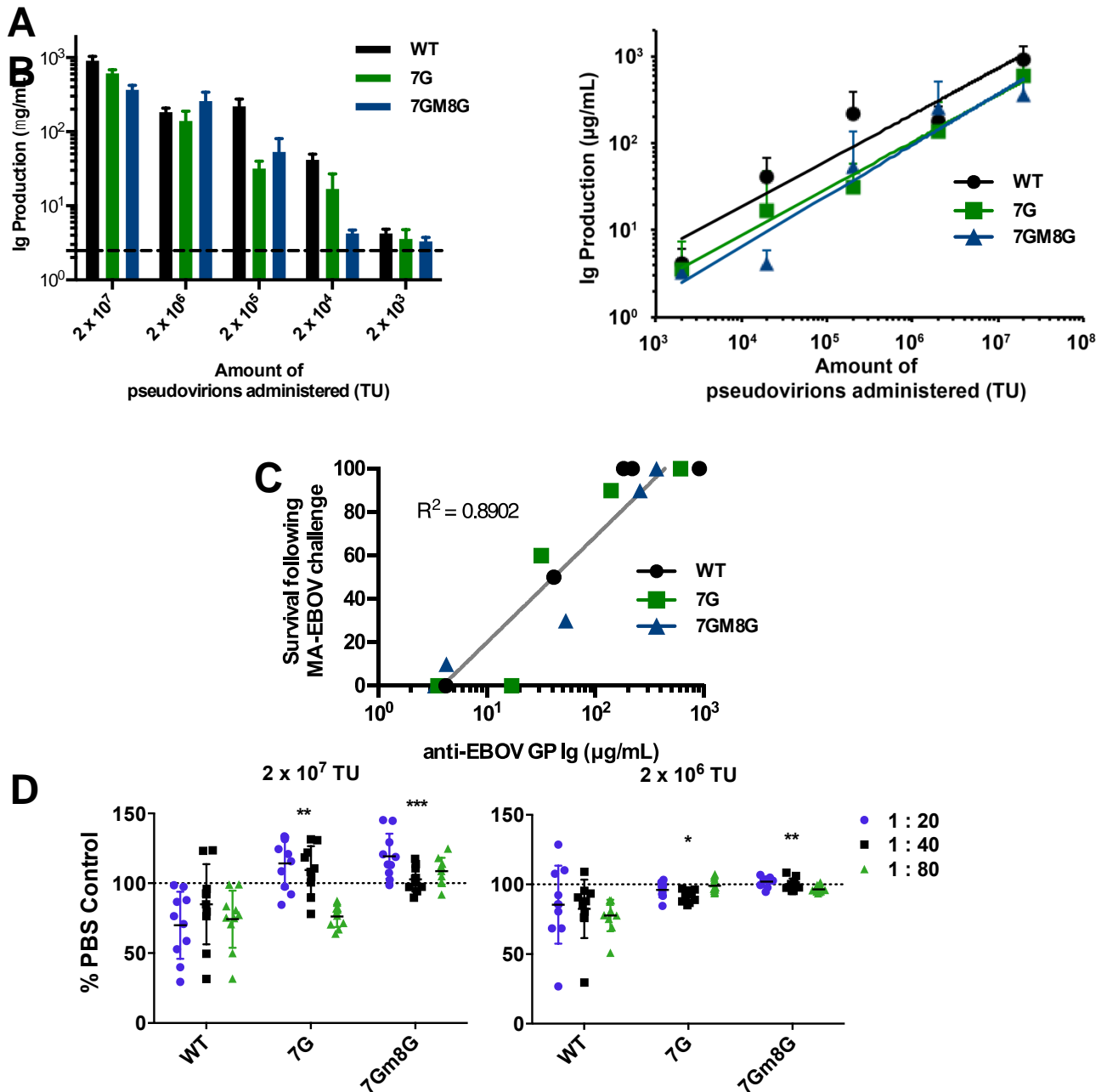
**Figure 4:** VSV pseudovirions bearing deglycosylated EBOV GP do not provide protection against SUDV challenge. C57bl/6 IFNAR<sup>-/-</sup> (n=10 mice) were primed and boosted with  $2 \times 10^7$  TU of the indicated VSV pseudovirions followed by challenge with (A) 1000 pfu of WT SUDV or (B) 1000 pfu of WT EBOV. Percentage of weight loss is shown at the indicated time following infection.



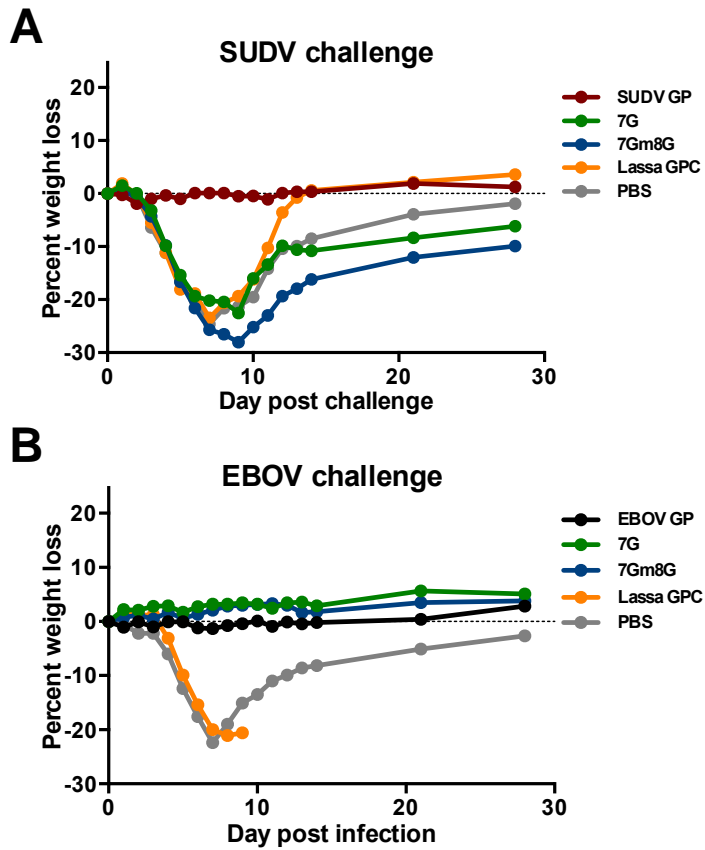
**Figure 1:** Vaccination/challenge regimen demonstrating protection against homotypic challenge. (A) Top-down view of models of EBOV GP and a GP mutant fully deglycosylated for GP1 N-linked glycans. RBD is shown in red, glycan cap is shown in teal, GP2 is shown in tan, N-linked glycans are shown in orange, and the MLD (not included in the crystal structure) is modeled as a gray sphere. Two N-linked glycans found on GP2 were not mutated in our glycosylation mutants investigated in these studies and are shown around the base of GP2. *Left*, WT GP; *right*, 7Gm8G mutant lacking all GP1 N-linked glycans indicated by the absence of orange glycans modeled on GP1 and an increase in transparency of the MLD. (B) Vaccination regimen. (C) Protection conferred by  $2 \times 10^7$  TU of EBOV GP/VSV pseudovirions ( $n = 9$ /group) upon challenge of BALB/c mice with 1000 PFU of MA-EBOV. Significance was determined by Mantel-Cox Test, \*\*  $p < 0.001$ . (D) Sickness score of surviving mice at indicated days post infection. Data are presented as average  $\pm$  SD.



**Figure 2:** Survival of C57bl/6 females vaccinated with the indicated transducing units (TU) of VSV lacking its native glycoprotein and pseudotyped with EBOV GP (black lines), 7G (green lines), or 7Gm8G (blue lines) (n= 10/treatment). Transducing units (TU) were determined by Vero cell transduction of VSV/EBOV GP; pseudovirion equivalent of mutant viruses were normalized to WT levels by the quantity of VSV matrix in each stock. Vaccinated mice were challenged with 100 pfu MA-EBOV and monitored for 28 days. For comparison, the PBS control is shown for each dosage. Survival of PBS control mice was compared to the other vaccine treatments with significance determined by Mantel-Cox Test, \* $p < 0.01$ , \*\* $p < 0.001$ .



**Figure 3:** Humoral responses in mice vaccinated with WT EBOV GP/VSV were more robust than that of mice vaccinated with deglycosylated pseudovirions. Sera were collected from vaccinated mice three weeks following boost. (A) Anti-EBOV GP Ig levels. Dashed line represents Ig levels observed in PBS-treated mice. (B) Correlation between the quantity of pseudovirus administered as a vaccine and Ig production. Trend line for each vaccine treatment is shown independently. (C) Correlation between group survival and anti-EBOV GP Ig in sera. Trend line is shown for pooled results from all three pseudovirion treatments. (D) Neutralizing activity present in vaccinated mice sera. Serum was serially diluted and incubated with EBOV GP/VSV for 30 minutes at 37°C prior to addition to Vero cells. Data are shown as transduction (GFP positive cells) presented as a percentage of PBS control. Significant differences in neutralization conferred by EBOV GP compared to either of the deglycosylated EBOV GP groups were determined by two-way ANOVA with Tukey post hoc analysis, \*  $p < 0.05$ ; \*\*  $p < 0.005$ ; \*\*\*  $p < 0.0001$ .



**Figure 4:** VSV pseudovirions bearing deglycosylated EBOV GP do not provide protection against SUDV challenge. C57bl/6 IFNAR<sup>-/-</sup> (n=10 mice) were primed and boosted with  $2 \times 10^7$  TU of the indicated VSV pseudovirions followed by challenge with (A) 1000 pfu of WT SUDV or (B) 1000 pfu of WT EBOV. Percentage of weight loss is shown at the indicated time following infection.